

# Heterozygosity of SNP513 in Intron 9 of the Human Calretinin Gene (*CALB2*) is a Risk Factor for Colon Cancer

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**Abstract.** *Background: The Ca<sup>2+</sup>-binding protein calretinin (CR), while absent in normal colonocytes, is expressed in the majority of poorly differentiated colon carcinomas, and is hypothesized that mutations in the distal part (from exon 7 to 10) of the CR gene (CALB2) could be responsible for the aberrant CR expression. Materials and Methods: Using PCR amplification of genomic DNA and restriction fragment length polymorphism analysis, four single nucleotide polymorphisms (SNPs) were identified in CALB2 intron 9. Results: A significant positive association between the genotype at SNP513 and colon cancer was found: more C/T heterozygotes were found in patients with colon tumors (60%) compared to healthy controls (35%) or patients with lung tumors (38%). Our results indicate that C/T heterozygosity at position 513 is linked to CR expression in colon tumors and colon cancer cell lines, suggesting a link with increased carcinogenesis. Conclusion: The SNP513 in human CALB2 may thus be a predictive marker for colon tumors.*

In Western countries, colorectal cancer is the second leading cause of cancer-related deaths and in many instances, the disease is diagnosed too late. Incurable cancer is detected in approximately one quarter of patients, and one half of patients who undergo surgery will ultimately develop metastases (1). Thus, an early detection method, when curative therapies are still effective, would be the most promising strategy. The 2000 guidelines of the American Cancer Society for the early detection of colon cancer

include the fecal occult blood test, flexible sigmoidoscopy and colonoscopy (2). Evaluation of molecular alterations in fecal DNA was proposed as a potential, noninvasive, alternative tool; mutations in p53 (*TP53*) and K-ras (*KRAS*) were detected in approximately one third of cases with a minimal or no co-presence of these gene alterations (3). Moreover, a null-mutation of the glutathione S-transferase T1 (*GSTT1*) results in a statistically significant increased risk for gastric cancer (4), which has been confirmed in a recent SNP interaction study (5). Hence, a better understanding of tumor biology at the molecular level would also help to identify novel determinants of response to chemotherapy in colon cancer (6) and/or to identify patients with the highest probability of responding to therapy.

Calretinin (CR) and the alternatively spliced form calretinin-22k (CR-22k) are members of a large family of Ca<sup>2+</sup>-binding proteins possessing respectively six and four (7) highly conserved Ca<sup>2+</sup>-binding motifs, which are referred to as EF-hand domains (8). CR is mainly expressed in specific neurons in the central and peripheral nervous system (9), and in several other organs, e.g. testis and ovary. The exact role played by CR in the various cell types remains unknown, but suggested functions include a general role as Ca<sup>2+</sup>-buffer in non-excitabile cells, an involvement in the excitability of neurons expressing this protein (10), and a function in neuroprotection against excitotoxicity (11, 12). CR is expressed in several colon adenocarcinoma cell lines (13) and also in primary colon tumors (14). Recently, antibodies against CR have gained much attention as specific and sensitive markers for mesotheliomas, tumors of the pleura of the lung, mainly of the epithelioid and the mixed type (15, 16). It is important to note that the smaller isoform CR-22k (M<sub>r</sub> 22 kDa) arising from deletion of exons 8 and 9 in the CR mRNA has been observed only in transformed tissue and derived cell lines (17, 18). Although the function of both isoforms in tumor cells is not yet clear, transfection of CR-negative colon carcinoma cells (Caco-2)

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with plasmids coding for CR or CR-22k increases their resistance towards the growth-inhibiting effect of butyrate (19), a substance known to induce the differentiation process in enterocytes. Conversely, treatment of CR-expressing human colon adenocarcinoma WiDr cells with butyrate leads to a down-regulation of CR (20).

Several factors affect splicing, the most obvious being mutations in the 5' or 3' branch sites, but also variations in the activity and/or quantity of general splicing factors may cause alternative splicing. The third mechanism is negative regulation by the binding of factors that inhibit splicing. We hypothesized that mutations in the 5' or 3' branch sites might be responsible for the alternative splicing. We thus sequenced the 3' region of exon 7, the 5' region of intron 7 and the region containing the junction intron 9/exon 10 of DNA that was isolated from normal tissue, colon cancer cell lines and primary colon cancer biopsies. Besides the canonical splice donor/acceptor sites, other intron motifs also appear to play a role in correct splicing. Introns are believed to play a role in gene regulation and in some cases enhance susceptibility to cancer (21). For instance, polymorphisms and germ line mutations in intron 6 of *TP53* (tumor suppressor gene) apparently predispose individuals with specific single nucleotide polymorphism (SNP) alleles to cancer (22-24). In addition, a mutation in intron 3 of the calpain gene (*CAPN1*) is associated with type 2 diabetes mellitus (25). Thus in addition to sequencing intron/exon junctions, we searched for SNPs in introns 7 and 9 of the human calretinin (*CALB2*) gene.

## Materials and Methods

**Samples.** Genomic DNA from 19 colon tumor samples was obtained from the University Hospital of Bern, Switzerland (Institute of Medical Oncology, Prof. M. Fey). Paraffin sections from an additional 57 colon tumors were obtained from R. Weimann (Department of Pathology, Inselspital, Bern: 35 samples) and J. P. Musy, (Kantonsspital Luzern: 22 samples, previously described (14)). All patients resided in or near the Canton of Bern. The average age of the patients when the samples were taken was  $65 \pm 14$  years. As a first control group, blood samples were used from 152 apparently healthy individuals who were considered to be eligible "healthy" blood donors for the Blutspendedienst in Bern and who resided in the same area as the patient group. To qualify as blood donors, participants had to be free of infection with HIV, HBV and HCV, not be IV drug users or have been diagnosed for any form of cancer. The large majority of individuals in both groups were Caucasian. The local ethical committee had approved the collection of these blood samples. The samples were anonymized; hence, tracing back to patients and blood donors was not possible. The second control group consisted of 50 samples of lung tumor DNA also received from the Department of Pathology, Inselspital, Bern (average patient age:  $64 \pm 8$  years). These samples were obtained from patients with the same geographic and ethnic background as those in the other two groups.

Supplemental Table S I. *CALB2* intron 9 SNPs of normal colon tissue and four colon carcinoma cell lines.

Cell line/Clone #	Pos. SNP	242 G/T	363 C/T	491 C/T	513 C/T
Human (DOE institute)*		G	T	T	C
normal colon/1		G	C	C	C
normal colon/6		G	C	C	C
normal colon/10		G	C	C	C
WiDr /1		G	C	C	T
WiDr /3		G	C	C	T
WiDr /6		G	C	C	C
Colo205/1		G	C	C	T
Colo205/2		G	C	C	T
Colo205/3		G	C	C	T
Co115-3/3		G	T	T	C
Co115-3/4		T	T	T	C
Co115-3/5		G	T	T	C
CaCo-2/2		G	T	T	C
CaCo-2/6		G	T	T	C
CaCo-2/7		G	T	T	C

\* An additional sequence in the Genebank by the DOE Joint Genome Institute (definition: *Homo sapiens* chromosome 16 clone CTD-2520I13; accession: AC106736, GI:1942485) was found to be identical to the *CALB2* genome sequence. Differences between the sequences were seen at four positions (first nucleotide of intron 9 is position 1). Genomic DNA was amplified by PCR, the fragments were cloned into a sequencing vector and DNA from three *E. coli* colonies/samples were sequenced.

The following cell lines were obtained from the American Type Culture Collection (ATCC): SW480, SW620, WiDr, HT-29, SW1116, Lovo, Colo205, Caco-2, SK-CO-1, LS180 and Met-5A. Co112 and Co115/3 cells were a gift of Dr. B. Sordat, Swiss Institute for Experimental Cancer Research, Epalinges, and the mesothelioma cell lines SPC212, SPC111, ZL55, ZL5, ZL34 were a gift from Dr. R. Stahel, University Hospital, Zurich, Switzerland. Details relating to the cell culturing conditions of the colon cancer and the mesothelioma cell lines are to be found in the literature (13, 26, respectively). Other cell lines included: K562 (chronic myeloid leukemia cells); KG1 (hematopoietic progenitor cells); MOLT-4 (lymphoblastic leukemia cells); HL-60 and KG1 (myelogenous leukemic cells), MDA-MB-453 (breast cancer cells); SKUT-1B (uterine tumor cells); Calu-1 (lung carcinoma cells); WI38 (lung embryonic fibroblast cells); IMR90 (embryonic fibroblasts); A549 (non-small cell lung cancer cells); SaOs-2 (osteosarcoma cells).

**Isolation of genomic DNA, PCR analyses, DNA sequencing and restriction fragment length polymorphism (RFLP) analyses.** Genomic DNA from different cell lines (Supplemental Table S I and Table I) and from paraffin sections was isolated using standard methods. Genomic DNA (0.5-1 µg) was used for the PCR analyses. For this, two regions in intron 9 of the *CALB2* gene were amplified. The

Table I. A) SNPs at positions 363, 491 and 513 of CALB2 intron 9 of various cell lines.

#	SNP pos. sample	363	491	513	CR*	#	SNP pos. sample	363	491	513	CR*
	normal colon	C/C	C/C	C/C	-						
1	<b>Caco-2</b>	C/T	C/T	C/C	-	15	<b>SK-CO-1</b>	C/C	C/C	<b>C/T</b>	+
2	K562	C/C	C/C	C/C	-	16	WI38	C/T	C/C	<b>C/T</b>	+
3	KG1	C/T	C/C	C/C	-	17	IMR90	C/T	C/C	<b>C/T</b>	+
4	HL-60	C/T	C/T	C/C	-	18	<b>Co112</b>	C/C	C/C	<b>C/T</b>	+
5	MDA-MB-453	T/T	T/T	C/C	-	19	<i>SPC212</i>	C/C	C/C	T/T	+
6	<b>SW1116</b>	C/C	C/C	T/T	-	20	<b>Colo205</b>	C/T	C/T	<b>C/T</b>	+
7	MOLT-4	C/C	C/C	T/T	-	21	A549	C/T	C/T	<b>C/T</b>	+
8	SKUT-1B	C/C	C/C	T/T	-	22	SaOs-2	C/T	C/T	<b>C/T</b>	+
9	ZL5	C/C	C/C	T/T	-	23	<i>SPC111</i>	C/T	C/T	<b>C/T</b>	++
10	<b>Lovo</b>	C/C	C/C	C/C	+/-	24	<b>Co115/3</b>	C/T	C/T	<b>C/T</b>	++
11	<b>SW480</b>	C/C	C/C	C/C	+/-	25	<b>LS180</b>	C/C	C/T	<b>C/T</b>	++
12	<b>SW620</b>	C/C	C/C	C/C	+/-	26	<b>HT-29</b>	C/C	C/T	<b>C/T</b>	++
13	Calu-1	C/C	C/C	T/T	+/-	27	ZL55	T/T	C/C	C/C	++
14	ZL34	C/C	C/C	T/T	+/-	28	<b>WiDr</b>	C/C	C/T	<b>C/T</b>	+++

\*Colon cancer cell lines are marked in bold and *mesothelioma* cell lines in italics. CR expression levels were determined by Western blot analyses and were qualitatively classified as follows: (-) no expression, (+/-) weak expression, (+) moderate expression, (++) strong expression and (+++) very strong expression. For the ANOVA analysis, the values 0, 0.5, 1, 2 and 3 were attributed to the above qualitative classifications, respectively. Values for colon cancer (13) and *mesothelioma* cells (26) were taken from previous reports and compared to the expression levels in the other cell lines (Western blots not shown). Heterozygous samples (C/T; bold) at position 513 are strongly correlated with elevated CR expression levels in all cell lines (one-way ANOVA;  $F_{2,25}=8.1$ ,  $p=0.002$ ); Analysis of seven CR-positive and five CR-negative colon cancer cells (bold) also show significant differences with respect to C/T heterozygosity at position 513 (Fisher's exact test ( $p=0.015$ )). The other two loci show no correlation between haplotype and CR expression (SNP 363:  $F_{2,25}=0.1$ ,  $p=0.92$ ; SNP 491:  $F_{2,25}=0.4$ ,  $p=0.66$ ).

B) Relative distribution of C/C, C/T and T/T at SNPs 363, 491 and 519 in cell lines with no (-) or low (+/-) CR expression (left panel) and in CR-positive (+ - +++) lines (right panel).

"Low" CR (cell lines 1-14)				"High" CR (cell lines 15-28)			
Intron position, restriction enzyme	Genotype	Number of lines	%	Intron position, restriction enzyme	Genotype	Number of lines	%
363, <i>MnII</i>	C/C	10	71.4%	363, <i>MnII</i>	C/C	6	42.9%
	C/T	3	21.4%		C/T	7	50%
	T/T	1	7.2%		T/T	1	7.1%
491, <i>MnII</i>	C/C	11	78.6%	491, <i>MnII</i>	C/C	8	57.2%
	C/T	2	14.3%		C/T	5	35.7%
	T/T	1	7.1%		T/T	1	7.1%
513, <i>NlaIII</i>	C/C	8	57.1%	513, <i>NlaIII</i>	C/C	1	7.1%
	C/T	0	<b>0%</b>		C/T	12	<b>85.8%</b>
	T/T	6	42.9%		T/T	1	7.1%

primer pair 244F and 427R and the pair 438F and 629R yielded fragments of 184 and 192 base pairs, respectively. The primer sequences were: 244F: ACTAGGGTGTGACCACGCTGT; 427R: GTTATGAAGAAAACACCGGAGT; 438F: GGTAACCTTTA AATAGCCCCCG; 629R: GGGTGTTCAGATCGTGTAATGA. The amplicons from several cell lines and from a control colon tissue sample were cloned in a plasmid vector by TOPO-Cloning (Invitrogen, LuBioScience GmbH, Lucerne, Switzerland) and sequenced (Microsynth AG, Balgach, Switzerland). For the RFLP analyses, PCR product fragment A was digested with *MnII* to check

for position 363, fragment B was digested with either *MnII* (pos. 491) or *NlaIII* (pos. 513). The digested fragments were separated either on 2% agarose or on 6% polyacrylamide gels, and the gels were stained with ethidium bromide. As controls for the RFLP analyses, PCR products from the cell line-derived clones (Table S I), where the genotype (C or T) at the given SNP was determined by DNA sequencing, were used as controls. For instance, clones 1 and 6 from WiDr cells have either a T or a C at SNP 513, respectively. Thus at this site, *NlaIII* cuts the PCR fragment B of clone 6 (C), but not of clone 1 (T).

**Western blot analysis and immunohistochemistry.** Soluble proteins from different cell lines were isolated as described elsewhere (27), separated by SDS-PAGE, transferred onto nylon membranes and probed with the antiserum CR7696 recognizing CR and CR-22k (28). Immunohistochemistry was performed on 39 non-small cell lung cancer (NSCLC) and 57 colon cancer samples using the same CR antibody and the same protocol as described elsewhere (18). The stained sections were analyzed by light microscopy by two observers not knowing the results from the genetic analysis. Each section was evaluated twice within a period of 3-6 months. The tumors were classified according to the staining intensity and the size of the immunoreactive area within the tumor mass. Based on the two parameters the values 0, 0.5, 1, 1.5 or 2 were attributed to each sample.

**Statistical analysis.** To compare the level of CR expression scores among SNP genotypes, the Kruskal-Wallis test, as implemented in the SAS statistical software (PROC NPAR1WAY; SAS Institute Cary, NC, USA) was used. This approach is more appropriate than ANOVA, since the expression scores are in arbitrary units and unlikely to be normally distributed. Additionally, we divided the cell lines into two categories: low expression (score <1) and high expression (score ≥1), and used Fisher's exact test to test for independence between this class assignment and SNP genotype; this test is a replacement for a  $\chi^2$  test when the sample size is small (29). To test for differences in genotype and allele frequencies between the groups of subjects (colon and lung cancer patients and healthy blood donors), we used both Pearson's  $\chi^2$  test and Fisher's exact test. Since the two tests yielded congruent results, only those from the  $\chi^2$  tests are reported.

## Results

The alternative splice product CR-22k has so far been detected only in tumor samples of the colon (30), in mesotheliomas (18) and in cell lines derived from these tumor types (17). Based on the hypothesis that mutations in the 5' or 3' branch sites cause the alternative splicing, we sequenced the 3' region of exon 7/ intron 7 and the 3' end of intron 9/ exon 10 from normal colon and primary colon cancer biopsies as well as from colon cancer cell lines (WiDr, Colo205, Caco-2, Co115/3). Both junctions were identical in all samples, excluding that alternative splicing in tumor samples (tissue and cell lines) was due to incorrect splice donor, splice acceptor and branch sites in introns 7 and 9 (Figure 1). Moreover, the 3' end of intron 7 and the 5' end of intron 9 were found to be unaltered between DNA from normal tissue and tumor cells. Since other motifs in intron sequences also seem to play a role in correct splicing, the entire sequences of *CALB2* introns 7 and 9 were analyzed. The intron 7 sequence (313 base pairs, Figure 1) from the same four cancer cell lines as above and from normal colon tissue were all identical. In contrast, the complete sequence of *CALB2* intron 9 (740 base pairs) revealed four SNP sites (Table S I). A C/T polymorphism at three of the sites

(pos. 363, 491 and 513) and a G/T polymorphism at position 242 were observed. The small number of sequences (three clones for each sample) did not allow us to determine the genotype with certainty at the various SNP positions. Hence, three of the SNPs were investigated by RFLP analysis. At positions 363, 491 and 513, sites for the restriction enzymes *MnlI* and *NlaIII* enabled us to ascertain whether a C or a T was present in the two alleles. Genomic DNA from the various cell lines was amplified by either PCR primer pairs 1 or 2 yielding fragments A or B of 184 and 192 base pairs, respectively (Figure 2). Fragment A contained SNP 363 (*MnlI*) and fragment B SNPs 491 (*MnlI*) and 513 (*NlaIII*). PCR fragments A and B were subsequently digested with the appropriate enzymes yielding different fragments that permitted an identification of the genotype (Figure 2).

There was no association between CR expression of tumor cell lines and the *CALB2* genotype at either SNP 363 or SNP 491 (Table I; Kruskal-Wallis test:  $p>0.5$  in both cases). In contrast, the expression of CR clearly differed among the SNP 513 genotypes (Kruskal-Wallis test,  $\chi^2=12.6$ ,  $df=2$ ,  $p=0.002$ ). The C/T heterozygotes were over-represented in lines with moderate to high expression (score ≥1, odds ratio (OR): 21.0, 95% confidence interval (CI): 2.9-151.4; Fisher's exact test:  $p<0.003$ ), while all nine lines with no CR expression were C/C or T/T homozygotes. Pair-wise comparisons indicated that tumor cell lines heterozygous for SNP 513 had on average significantly higher CR expression levels than both C/C homozygotes (Kruskal-Wallis test:  $\chi^2=8.8$ ,  $df=1$ ,  $p=0.003$ ) and T/T homozygotes ( $\chi^2=8.7$ ,  $df=1$ ,  $p=0.003$ ). There was no difference between the two homozygous genotypes ( $\chi^2=0.01$ ,  $df=1$ ,  $p=0.95$ ). These results also held true when only colon cancer lines (bold in Table I) were compared. The seven C/T cancer lines had significantly higher expression scores than the four C/C colon cancer lines ( $\chi^2=6.0$ ,  $df=1$ ,  $p=0.015$ ) and C/T lines had a significantly lower probability of having low or no CR expression (Fisher's exact test:  $p=0.015$ ).

These data indicate that the heterozygous genotype at SNP 513 may be linked to calretinin expression, which in turn is associated with colon tumors (14). We thus set out to test the relationship between the SNP genotype at position 513 (as well as at positions 363 and 491) and susceptibility to colon cancer using genomic DNA from 76 colon tumor samples. As a first control group, we used blood samples from 152 apparently healthy individuals (males and females of various ages with a similar ethnic background to the colon tumor patients) who, based on their general health status, were eligible for donating blood. To exclude the possibility that C/T heterozygosity is non-specifically associated with any tumor type, 50 lung cancer samples were also analyzed.



**A** Exon.7.....gatggcaaattgggcctctcagagatgtcccgGTAAGCACCTCACCCCCGGGGTCACTGAT  
 ACTGGCTCCCACAGGTCATTCCTGTGTTATCCGTCCTGAGATCCATTGGTGGGA  
 AAGTGACAGGTGCGGGTGTCAAGAAGCTCAAGACAAAGCAAGATAGAATTGTGA  
 CCGTCAACACCTCACCTTGTCTGTCTCCCTCGTTTTTGAACCTCCCACTGATTCAAT  
 ATGTGTGAAGTGCTCAGAAATCATTTCTGTAAGTGCAGGCACCGCCTCTGCCTTC  
 CCCTCTCCCGCTTGCCCTTGCCCTGTGCAGTCTCCTCATCTCTGCTCT**TAAC**ATTTTC  
 TCCCCAGactcctgcctgtccagaaaacttctgtcttaa....exon 8

**B** Exon.9.....cttcacattttacgacaagGTAAGAGAGGGAGTTGGCATGGCAGGGAAAAATCAGAA  
 GCCCATCAGCCCGTCCAGAAGGGCTCAGCTTCATCCCTGGGAAGAGACAGCTTTC  
 CAGGGGTGGCCTGGGCCGTGTGGTTTCTTCTGCCGCATCTTCTGCTGTATGAGA  
 AGGCAAATGTCATTCTCCACCGGTGGCCTATGGAGCCCAAGGGGTTGGTTTCTGC  
 AGAGTGACGCCGAGAATCGTTGGGGGAGGACTATGCTTAGAACTAGGGTGTGACC  
ACGCTGTCGGGAGCCAAAGGGAAGAGACACTCAGAACTGCCCTGGTGCCAGATC  
 ACAATTCTGCCCAGGGCCAAGTCTTTCTCTGGGAAGTTGGAAGTTAGATGA[CCTC]  
 CATACCCACCCCTCCCTGGGCTGTCCCTGCCACATGACTCCGGTGGTTTTCTTCA  
 TAACCAGTGTTGGAGGTAACTTTAAATAGCCCCCGGACTCAGGGAGTTAACCAA  
 TGCTTCTTGAA[CCTC]ACTTAAATTTTCAACGCA[CATG]AAAAGCACCACAATGAAA  
 GGCTACCCAAAGCTTGCACCCACTGCCACCTTCCTGC[CATG]ACTGGTTAAGGCAG  
 AAGGGACACATTATTTTGTCATTACACGATCTGAACACCCCTTTTGCACAGAGTAA  
 TGGAGAGGCTAGACTCTTAGACATCCCTGGAATGGGTCGGGACAGCAGGGGCC  
 AGGCTTTAGAGCTCTGGGTTGACTCTGCTCCCATCCCCAGgatagaagcggctacattgacga  
 gcacc....Exon 10

Figure 1. A) Genomic DNA sequence of CALB2 intron 7 from normal colon tissue and four colon carcinoma cell lines. DNA sequences from all samples were identical. Splicing donor and acceptor sites: 5' GT (U in the RNA) and 3' AG are underlined. Exon sequences are given in small capital. The splicing consensus sequence CTCTAAC (bold) confers to the expected sequence (Py<sub>80</sub>NP<sub>80</sub>Py<sub>87</sub>Pu<sub>75</sub>AP<sub>95</sub>) which is present 18-40 bp upstream of the 3' splice site (Pu: G, A/Py C, T). B) Genomic DNA sequence of CALB2 intron 9 from normal colon tissue. Splicing donor and acceptor sites: 5' GU and 3' AG are underlined. Exon sequences are given in small capital. The splicing consensus sequence GGTGAC (bold) confers to the expected sequence with the exception of the first nucleotide (underlined), which is most often Py, but in CALB2 intron 9 is G. Sequences of primers 244F and 427R yielding a PCR fragment of 184 bp are given in italics. Primers 438F and 629R (PCR fragment size: 192 bp) are given in italics and underlined. Boxed sequences indicate restriction sites for MnlI (CCTC) and NlaIII (CATG); the SNPs within the boxes are marked in bold.

The frequencies of the three genotypes (C/C, C/T, T/T) at position 513 differed between the colon cancer patients (n=76) and the healthy control group ( $\chi^2=13.7$ ,  $df=2$ ,  $p<0.0011$ ), and between the colon and lung cancer patients ( $\chi^2=8.4$ ,  $df=2$ ,  $p=0.015$ ); there was no difference between the healthy controls and lung cancer patients ( $\chi^2=3.1$ ,  $df=2$ ,  $p=0.21$ ). These last two groups were at Hardy-Weinberg equilibrium with respect to SNP 513 (healthy subjects:  $\chi^2=0.1$ ,  $df=1$ ,  $p=0.78$ ; lung cancer patients:  $\chi^2=2.75$ ,  $df=1$ ,  $p=0.09$ ). In contrast, the samples of colon cancer patients showed a significant excess of heterozygotes over the Hardy-Weinberg expectation ( $\chi^2=10.5$ ,  $df=1$ ,  $p=0.0012$ ). In a direct comparison, the C/T genotype was more frequent among colon cancer patients (60%) than between the healthy controls (35%) and the lung cancer patients (38%). The frequency of the C/C homozygotes showed an opposite pattern (37% in colon cancer patients *versus* 62% and 59% in lung cancer patients and healthy controls, respectively).

The C/T:C/C OR between colon cancer patients and controls was 2.8 (95% CI: 1.6-5.0,  $\chi^2=12.4$ ,  $df=1$ ,  $p=0.0004$ ); the analogous OR between colon and lung cancer patients was 2.7 (95% CI: 1.3-5.6,  $\chi^2=7.0$ ,  $df=1$ ,  $p=0.0082$ ). The T/T homozygotes were too rare to allow a meaningful comparison between samples. The frequency of allele T among the colon cancer patients was 0.33 and was significantly higher than among the healthy controls (allele frequency: 0.23,  $\chi^2=4.7$ ,  $p=0.030$ ) and the lung cancer patients (allele frequency: 0.19,  $\chi^2=5.9$ ,  $p=0.016$ ); there was no indication of a difference between healthy and lung cancer samples ( $\chi^2=0.8$ ,  $p=0.36$ ).

For a subset of 57 colon tumor and 39 lung cancer cases, we also had access to tissue paraffin sections, which we stained for CR (Tables IIA and III). Of the 57 tissue sections from colon cancer patients, 44 (77%) were considered to be CR-immunoreactive (defined as an IHC score >0.25), a value that is in line with previous findings

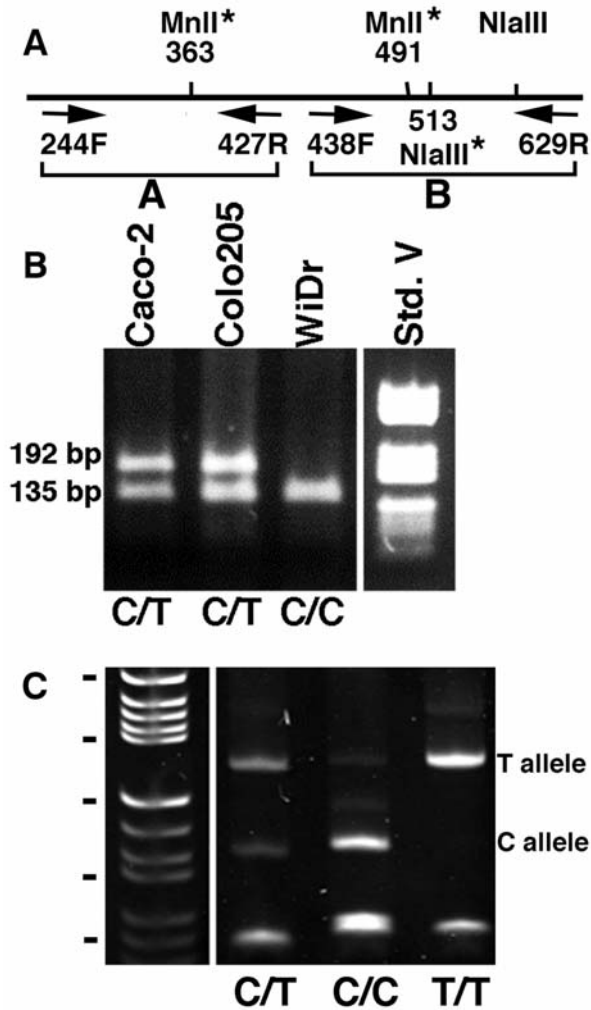


Figure 2. Haplotype analysis in *CALB2* intron 9 at SNP 491 and at SNP 513. A) Schematic diagram of *CALB2* intron 9 with primers used for PCR amplification yielding fragments A and B. Fragment A is digested with *MnlI* (reveals haplotype at position 363) and fragment B with either *MnlI* or *NlaIII* (positions 491 and 513, respectively). SNP sites are marked with \*. B) Digestion of PCR fragment B (192 bp) with *MnlI* results in two fragments of 135 and 57 bp, if a C is present at position 491. C) Digestion of fragment B with *NlaIII* amplified from three healthy control samples representing the three genotypes C/T, C/C and T/T. Digestion results in three fragments (79, 59, 54 bp) in the case of C (middle lane) and in two fragments (138 and 54 bp) in the case of T (right lane). Samples were run on 6% PAA gels for improved resolution. The upper faint bands in the middle lane (C/C) are the result of incomplete digestion. In B) and C), DNA marker V (Roche) was used. Sizes of fragments are (in bp) from top to bottom: 267, 234, 213, 192, 184, 124, 123, 104, 89, 80, 64, 57. Underlined fragments are marked by bars in C.

where approximately 60-70% of GIII colon tumors were found to be CR-immunoreactive (14). The average IHC score determined semi-quantitatively for the samples with the C/T genotype was slightly higher than for the C/C genotype ( $0.89 \pm 0.55$ ,  $n=33$  vs.  $0.70 \pm 0.45$ ,  $n=22$ ;

average  $\pm$  SEM), but the difference was not statistically significant. Furthermore, no pathological characteristics, in particular grading ( $p=0.61$ ;  $\chi^2$ -test) or stage ( $p=0.18$ ) were associated with the SNP 513 polymorphism status. The percentages of GI, GII and GIII tumors with C/C (14%, 50% and 36%) were practical identical to the ones in the C/T group (15%, 52% and 33%). As shown before (14, 30), in some samples, cells of the tumor mass were distinctly CR-positive, while in other samples mainly giant fibroblastic cells were stained by the CR antiserum and crypt cells in these samples were largely CR-negative (not shown). The fibroblastic cells were always restricted to particular regions of the tumor, often surrounding the tubular organotypical tumor formations.

No significant differences were detected in genotype or allele frequencies at the other two SNP sites (363 and 491; Table IIB) between the healthy and the colon tumor patient groups (all  $p>0.19$ ). This indicates that it is specifically SNP 513 that is differently distributed in colon tumors vs. healthy controls or lung tumors. In contrast, the lung cancer patients carried more often T/T and less often C/C at SNP 363 than either the healthy group ( $p=0.006$ ) or the colon cancer patient group ( $p=0.010$ ). This was reflected in the higher frequency of allele T at SNP 363 among the lung cancer patients (0.40) than either among healthy individuals (0.27,  $p=0.016$ ) or the colon cancer patients (0.22,  $p=0.008$ ).

Therefore, C/T heterozygosity at *CALB2* SNP 513 might be a risk factor and/or a predictive marker for colon tumors. Evidently, this statement is only valuable if the C/T heterozygosity is not the result of a spontaneous mutation in the tumor tissue itself. In a relatively small number of samples ( $n=4$ ), we had access to genomic DNA from the tumor and normal tissue of the same patient. The SNPs at all four loci tested were always identical, irrespective of the tissue origin (tumor vs. normal). This indicates that these SNPs are not the result of somatic mutations occurring during tumorigenesis, but are germ line polymorphisms.

## Discussion

Genetic alterations, in particular loss of tumor suppressor genes including *APC* (adenomatous polyposis coli), *DCC* (deleted in colon carcinoma) and *TP53* (31) are observed in >70% of colon tumors, but also the up-regulation of oncogenes such as *K-ras* or *myc* is typical. A null mutation in glutathione *S*-transferase T1 (*GSTT1*), an enzyme implicated in the detoxification of carcinogens and subsequently preventing DNA damage, is considered as a risk factor in increasing the susceptibility to gastric cancer (4, 5).

Deregulated CR expression is seen in colon carcinomas (14) and in the many CR-positive colon cancer cell lines (13). While CR expression is observed in essentially all mesotheliomas of the epithelioid and mixed type (15, 16, 32),

Table II. A) SNP 513 analysis of genomic DNA from healthy controls (Ctrl; n=152) or from colon tumor (n=76) and lung tumor (n=50) samples.

		Colon tumor					Lung tumor			
		CR+	CR-*	CR-**	CR n.d.	Total	CR+	CR-	CR n.d.	Total
C/C	90 (59%)	16	3	3	6	28 (37%)	12	13	6	31 (62%)
C/T	53 (35%)	27	2	4	13	46 (60%)	3	11	5	19 (38%)
T/T	9 (6%)	1	1	0	0	2 (3%)	0	0	0	0 (0%)
Total	152 (100%)	44	6	7	19	76 (100%)	15	24	11	50 (100%)

Tumor samples were additionally grouped according to their CR expression status: CR-positive samples (CR+); CR-negative samples (CR-); samples in which the CR status was not determined (n.d.). In the latter category, genomic DNA, but no tumor tissue was available for IHC. \* IHC score 0.25; \*\* IHC score 0

B) SNP 363 and 491 analyses.

SNP Genotype	MnII (363)			MnII (491)		
	Ctrl	Colon	Lung	Ctrl	Colon	Lung
C/C	83 (55%)	26 (59%)	21 (42%)	112 (74%)	34 (63%)	30 (60%)
C/T	57 (37%)	17 (39%)	18 (36%)	35 (23%)	19 (35%)	16 (32%)
T/T	12 (8%)	1 (2%)	11 (22%)	5 (3%)	1 (2%)	4 (8%)
Total	152	44	50	152	54	50

Amongst the colon samples (n=54), 10 were excluded for genotype analysis at position 363, since the genotype could not be determined conclusively.

CR expression in colon cancer is clearly linked to the degree of tumor differentiation. Whereas only a small percentage (6%) of GI tumors are CR-immunoreactive, almost 67% of GIII tumors express this protein (14), suggesting that CR expression is increased as tumor cells dedifferentiate. This correlates with higher CR expression levels *in vitro* in poorly differentiated colon carcinoma cells with a high proliferation rate (13). Alternative splicing of the CR mRNA restricted to tumor cells *in vitro* and *in vivo* (18, 30) gives rise to the splice forms CR-20k and CR-22k (33). This finding prompted us to search for putative mutations in the *CALB2* gene in the region from exon 7 to exon 10, where the alternative splicing event occurs. No differences in the splice donor or acceptor sites were evident between colon cancer cells and normal colon tissue. However, four SNPs were detected in the *CALB2* intron 9 sequence. Three of these SNPs (position 363, 491 and 513) were accessible for RFLP analysis. In cell lines, a clear association existed between CR expression and C/T heterozygosity at SNP 513. Higher CR expression levels were detected in cell lines with the 513 C/T genotype and this was validated by comparing all 28 cell lines as well as the 12 colon cancer lines. Interestingly, all cell lines with confirmed CR-22k expression (WiDr, HT-29, LS-180, COLO205 and Co115/3 (17)) display the 513 C/T genotype (Table IA).

Hence, the question was raised as to whether the 513 C/T genotype is also over-represented in primary colon cancer specimens retrieved during surgery. Clearly the frequency of C/T heterozygotes was significantly higher in the colon cancer patient group compared to the healthy control group and lung tumor group. A good association existed between CR-immunoreactive colon tumors with SNP 513 C/T (27/46; 59%) and CR-immunoreactive colon cell lines (7/12; 58%), even though the latter were rather small in number. Heterozygosity is the most likely reason for the excess of C/T heterozygotes in the colon cancer group, but the overall number of T/T homozygotes was too small to entirely exclude the possibility that the increased CR expression is due to a dominant effect of the T allele. An argument against the latter point is the fact that 6 out of the 7 cell lines with T/T at position 513 had no (4) or weak (2) CR expression. The SNPs at position 363 and 491 did not differ between the colon cancer group and the healthy control group. Interestingly, T/T homozygotes with SNP 363, and the T allele generally, were significantly more frequent in lung tumor patients and might thus serve as an additional predictive marker. A more detailed analysis of this haplotype at SNP 363 needs to be conducted.

Table III. *CALB2* genotype at SNP 513 of 76 colon tumors (primary or metastatic), pathological grade (grade of tumor tissue differentiation) and analysis of 57 samples for CR expression by semi-quantitative immunohistochemistry (IHC).

Case	Grade of diff.	Genotype	IHC *	Case	Grade of diff.	Genotype	IHC*
1	2	<b>C/T</b>	<b>0.75</b>	39	1	<b>C/T</b>	<b>0.5</b>
2	2	C/C	0.5	40	1	<b>C/T</b>	<b>2</b>
3	3	<b>C/T</b>	<b>0.75</b>	41	3	C/C	0.25
4	2	<b>C/T</b>	<b>1</b>	42	3	C/C	0.75
5	2	<b>C/T</b>	<b>0.75</b>	43	2	<b>C/T</b>	<b>1</b>
6	2	C/C	0.75	44	1	<b>C/T</b>	<b>0.75</b>
7	3	<b>C/T</b>	<b>0.25</b>	45	1	T/T	1.75
8	3	<b>C/T</b>	<b>0.75</b>	46	1	C/C	0.75
9	3	<b>C/T</b>	<b>0</b>	47	1	C/C	0.75
10	2	T/T	0.25	48	1	C/C	0.5
11	2	C/C	1	49	2	<b>C/T</b>	<b>1</b>
12	2	<b>C/T</b>	<b>1</b>	50	3	<b>C/T</b>	<b>0</b>
13	2	C/C	0	51	2	C/C	1
14	3	C/C	0.75	52	2	C/C	0.25
15	3	C/C	0.75	53	3	<b>C/T</b>	<b>0</b>
16	3	<b>C/T</b>	<b>1</b>	54	2	<b>C/T</b>	<b>1.75</b>
17	3	C/C	1	55	2	<b>C/T</b>	<b>2</b>
18	2	C/C	0.75	56	1	<b>C/T</b>	<b>0.75</b>
19	3	<b>C/T</b>	<b>0.5</b>	57	1	<b>C/T</b>	<b>0</b>
20	2	<b>C/T</b>	<b>1</b>	58	n.d.	<b>C/T</b>	n.d.
21	2	C/C	0	59	n.d.	<b>C/T</b>	n.d.
22	2	<b>C/T</b>	<b>0.75</b>	60	n.d.	<b>C/T</b>	n.d.
23	3	C/C	1	61	n.d.	C/C	n.d.
24	2	<b>C/T</b>	<b>0.25</b>	62	n.d.	<b>C/T</b>	n.d.
25	2	C/C	0	63	n.d.	<b>C/T</b>	n.d.
26	3	C/C	0.25	64	n.d.	C/C	n.d.
27	2	<b>C/T</b>	<b>0.75</b>	65	n.d.	C/C	n.d.
28	2	<b>C/T</b>	<b>1</b>	66	n.d.	C/C	n.d.
29	2	<b>C/T</b>	<b>0.75</b>	67	n.d.	<b>C/T</b>	n.d.
30	2	C/C	0.75	68	n.d.	<b>C/T</b>	n.d.
31	3	<b>C/T</b>	<b>1</b>	69	n.d.	<b>C/T</b>	n.d.
32	3	C/C	1	70	n.d.	<b>C/T</b>	n.d.
33	3	<b>C/T</b>	<b>1</b>	71	n.d.	<b>C/T</b>	n.d.
34	3	<b>C/T</b>	<b>1</b>	72	n.d.	<b>C/T</b>	n.d.
35	2	C/C	0.5	73	n.d.	<b>C/T</b>	n.d.
36	2	<b>C/T</b>	<b>0.5</b>	74	n.d.	C/C	n.d.
37	2	<b>C/T</b>	<b>1.5</b>	75	n.d.	C/C	n.d.
38	2	<b>C/T</b>	<b>0.75</b>	76	n.d.	<b>C/T</b>	n.d.

For each tumor, the tumor grade (pathological classification) and the semi-quantitative immunohistochemical analysis (IHC) in two independent evaluations were performed at an interval of 3 months. The tumors were classified according to the staining intensity and the size of the immunoreactive area within the tumor mass. Based on the two parameters the values 0, 0.5, 1, 1.5 or 2 were attributed to each sample. The average for the two analyses is shown. 33/57 (58%) of the colon tumors analyzed by IHC and 46 of a total of 76 samples (60%) were heterozygous (C/T; bold) at SNP 513. From samples 58-76, only genomic DNA was available and neither the grade nor the IHC score could be determined (n.d.).

Mutations in chromosome 16 at the locus of *CALB2* (Locus: 16q22.2) have been described recently. The gene closest to the *CALB2* gene locus that is linked to colon cancer is that for E-cadherin (*CDH1*; Locus 16q22.1). E-cadherin participates in the regulation of cellular adhesion and motility, interacts with  $\beta$ -catenin and might act as a tumor suppressor through the Wnt signaling pathway. Since loss of one *CDH1* or of the related gene cadherin-11 (*CDH11*) allele was detected only in a minority of cases, the authors concluded that the two genes are only indirectly involved in the pathogenesis of colorectal cancer

(34). However in another study, LOH at 16q22.1 was detected in 75% (out of 50) of gastric tumors (35) and reduced expression levels of E-cadherin and  $\beta$ -catenin were identified at frequencies of 42% and 28%, respectively. The authors hypothesized that alterations in E-cadherin and  $\beta$ -catenin play a role in the initiation and progression of gastric cancer. Subsequently, the effects of changes in the expression levels of E-cadherin and CR work in an antagonistic way: down-regulation of the former and *de novo* expression of CR in human colon cancer cells favor the progression of these tumors



and are probably independently regulated. However butyrate, a known inducer of differentiation in enterocytes has been shown to enhance the expression of E-cadherin (36) and at the same time to down-regulate CR expression *in vitro* (19, 37). The decrease in CR expression is linked to the differentiation status of colon carcinoma cells, since in HT-29 cells subjected to glucose starvation, the CR expression levels and differentiation were markedly lower than in untreated cells (38).

How might particular SNPs be associated with increased susceptibility for certain tumor types? Obviously, missense mutations in the coding region of a gene may lead to subtle alterations in structure and modify/inhibit the interaction with targets (DNA, proteins). Several examples of this type have been shown for *TP53* missense mutations (39). More and more, mutations in non-coding regions including 5' untranslated regions (UTR), 3' UTR (40) and intronic sequences (41, 42) seem to be highly linked to certain tumor types. Interestingly, an over-representation of heterozygotes was often associated with the tumor phenotype. Yet only in rare cases was the SNP genotype correlated with the expression levels of the particular gene product. Since intronic sequences in tumor suppressor genes (*e.g.* *TP53*) are implicated in gene expression and DNA-protein interactions (23), the fact that heterozygosity is linked to the tumor phenotype in the above examples hints that subtle gene-dosage effects may be involved.

Our results indicate that specifically heterozygosity at SNP 513 of the *CALB2* gene, and not one of the homozygote genotypes (C/C or T/T), appears to be associated with elevated CR levels in colon tumors. In previous studies, SNP heterozygosity was reportedly associated with an increased risk for specific cancers. An increased heterozygote frequency was observed in: (i) the 3' UTR of the *CDK2A* gene coding for the two gene products p16<sup>INK4a</sup> and p14<sup>ARF</sup> in sporadic primary melanomas (40), (ii) intron 2 of the calcium channel subunit *CACNA2D2* in small cell lung cancer (41), (iii) intron 2 of *CDKN1A* (p21<sup>WAF1/Cip1</sup>), a downstream effector of p53 in breast cancer (42), and (iv) exon 18 (a silent C/T transition) of the candidate oncosuppressor gene *RAD54L* in human meningiomas (43). In contrast to our study, neither the mRNA nor protein levels of the affected genes were measured in the previous investigations. Thus, it is generally unknown whether heterozygosity at a particular SNP is linked to altered protein expression, as in our case, where CR protein levels were found to be elevated in colon tumors with the heterozygous (C/T) genotype. CR expression *per se* is probably not directly pro-oncogenic in colon cells. Hence, CR cannot be viewed as a classical proto-oncogene. However, CR-containing colon cells are more resistant to signals that induce their differentiation (19). CR expression could thus facilitate their acquirement of additional mutations, which would lead to a more malignant, dedifferentiated phenotype.

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